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**RENAL AND BLOOD PRESSURE RESPONSE TO A HIGH-SALT DIET IN MICE WITH
REDUCED GLOBAL EXPRESSION OF THE GLUCOCORTICOID RECEPTOR**

Short title: Glucocorticoids and Hypertension

Jessica R. Ivy*, Louise C. Evans*, Rebecca Moorhouse, Rachel V. Richardson, Emad A.S. Al-Dujaili
Peter W. Flatman, Christopher J. Kenyon, Karen E. Chapman, Matthew A. Bailey

University/British Heart Foundation Centre for Cardiovascular Science, The University of Edinburgh,
Scotland, UK

- JRI and LCE contributed equally to the experimental work in this paper

LCE: current address: Department of Physiology, Medical College Wisconsin

Correspondence: Matthew Bailey, PhD
Centre for Cardiovascular Science
The University of Edinburgh Medical School
The Queen's Medical Research Centre
47, Little France Crescent
Edinburgh
EH16 4TJ

Telephone/Fax: +44 1312429233/+441312426782

Email: matthew.bailey@ed.ac.uk

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25 **ABSTRACT(250/250)**

26 Salt-sensitive hypertension is common in glucocorticoid excess. Glucocorticoid resistance also presents
27 with hypercortisolaemia and hypertension but the relationship between salt intake and blood pressure (BP)
28 is not well defined. GR^{βgeo/+} mice have global glucocorticoid receptor (GR) haploinsufficiency and
29 increased BP. Here we examined the effect of high salt diet on BP, salt excretion and renal blood flow in
30 GR^{βgeo/+} mice.

31 Basal BP was ~10mmHg higher in male GR^{βgeo/+} mice than in GR^{+/+} littermates. This modest increase was
32 amplified by ~10mmHg following a high-salt diet in GR^{βgeo/+} mice. High salt reduced urinary aldosterone
33 excretion but increased renal mineralocorticoid receptor expression in both genotypes. Corticosterone, and
34 to a lesser extent deoxycorticosterone, excretion was increased in GR^{βgeo/+} mice following a high-salt
35 challenge, consistent with enhanced 24h production. GR^{+/+} mice increased fractional sodium excretion and
36 reduced renal vascular resistance during the high salt challenge, retaining neutral sodium balance. In
37 contrast, sodium excretion and renal vascular resistance did not adapt to high salt in GR^{βgeo/+} mice, resulting
38 in transient sodium retention and sustained hypertension. With high-salt diet, *Slc12a3* and *Scnn1a* mRNAs
39 were higher in GR^{βgeo/+} than controls, and this was reflected in an exaggerated natriuretic response to
40 thiazide and benzamil, inhibitors of NCC and ENaC, respectively.

41 Reduction in GR expression causes salt-sensitivity and an adaptive failure of the renal vasculature and
42 tubule, most likely reflecting sustained mineralocorticoid receptor activation. This provides a mechanistic
43 basis to understand the hypertension associated with loss-of-function polymorphisms in GR in the context
44 of habitually high salt intake.

45 **Keywords:** hypertension, renal, sodium, steroids, aldosterone, thiazide, ENaC,
46 mineralocorticoid

47

48 INTRODUCTION

49 Salt-sensitive blood pressure (BP) is an independent risk factor for both cardiovascular and renal disease
 50 and the salt-induced rise in BP reflects underlying renal (Hall, 2016) and vascular (endothelial) dysfunction
 51 (Morris et al., 2016). The failure of neural and hormonal regulatory systems to adapt to high dietary salt
 52 intake is an important contributing factor to salt-sensitivity. In this context, sustained glucocorticoid excess,
 53 whether overt in Cushing's or covert in Metabolic Syndrome, induces salt-sensitive hypertension and
 54 increases cardiovascular risk (Bailey, 2017).

55 How glucocorticoids induce salt-sensitive hypertension is not clearly understood. Several groups, including
 56 ours, have used animal models to examine the relationship between abnormal glucocorticoid activity and
 57 BP. For example, both modest glucocorticoid excess (Bailey et al., 2009; Mu et al., 2011) and impaired
 58 glucocorticoid metabolism (Bailey et al., 2011; Craigie et al., 2012; Mullins et al., 2015) cause salt-sensitive
 59 hypertension. Abnormal activation of the thiazide-sensitive cotransporter (NCC) (Velazquez et al.,
 60 1996; Ivy et al., 2016) and the epithelial sodium channel (ENaC) (Gaeggeler et al., 2005; Bailey et al., 2009)
 61 both contribute to the rise in BP. More subtly, clamping corticosterone in the mid-physiological range
 62 ablates the normal diurnal rhythmicity, activates NCC and induces non-dipping BP in mice (Ivy et al.,
 63 2016). Interestingly, adrenalectomy also induces non-dipping BP in mice (Sei et al., 2008) and in humans
 64 such circadian abnormalities have a detrimental impact on cardiovascular health (Henley and Lightman,
 65 2014).

66 In the current study, we turned our focus from models of glucocorticoid excess to a model of glucocorticoid
 67 resistance. In humans, glucocorticoid resistance (OMIM 615962) induces hypercortisolaemia but patients
 68 are not Cushingoid due to loss of function mutations in NR3C1, the gene encoding the glucocorticoid
 69 receptor (GR). In this study, we used a transgenic mouse where *Nr3c1* was disrupted by gene-trap
 70 integration of a β^{geo} reporter. This created a fusion protein lacking the ligand binding domain and thus
 71 rendered transcriptionally inactive (Michailidou et al., 2008). Homozygous transgenic mice ($\text{GR}^{\beta\text{geo}/\beta\text{geo}}$)
 72 die postnatally but heterozygous transgenics ($\text{GR}^{\beta\text{geo}/+}$) survive to adulthood and mRNA expression for GR
 73 in the brain, adrenal gland, liver, fat and muscle is reduced by 40-50% (Michailidou et al., 2008). In that
 74 study mice were fed rodent chow containing 0.25% sodium. In humans, daily sodium intake habitually

exceeds the recommended upper limits (Powles et al., 2013) and the interaction between dietary salt, genetic abnormalities in GR and BP homeostasis is not well defined. In this study we therefore exposed GR^{βgeo/+} mice to a high salt diet, revealing salt-sensitive hypertension and renal tubular and vascular abnormalities.

METHODS

Mice were backcrossed onto the C57BL/6J background for >8 generations. In the current study, all experiments were performed on male GR^{βgeo/+} mice aged 12-16 weeks using GR^{+/+} littermates as controls. Experiments were performed under a UK Home Office License, following ethical review by the University. Mice were fed either a standard diet (0.25% Na, RM1) or a high-sodium diet (2.5% Na diet 829504), both manufactured by Special Diets Services Ltd. (Essex, UK). All materials were obtained from Sigma-Aldrich UK unless otherwise stated.

Blood pressure was measured in a cross-sectional study in mice fed 0.25% Na diet (GR^{βgeo/+} n=16; GR^{+/+} n=19) or 2.5% Na diet (GR^{βgeo/+} n=15; GR^{+/+} n=17). The carotid artery was cannulated under thiobutabarbital anaesthesia (Inactin; 120 mg/kg IP) and after a 40min stabilisation period, mean arterial BP was recorded over 30-40min and an average taken for each mouse. In a separate cohort of mice, BP under basal conditions (0.25% Na intake) was measured by radiotelemetry in GR^{βgeo/+} (n=6) and control (n=4) mice, as described (Ivy et al., 2016). Mice were anesthetised with isoflurane (2% in O₂ for induction; 1.5% for maintenance; IsoFlo, Zoetis UK Limited, UK) and PAC-10 radiotelemeter devices (Data Sciences International, USA) were inserted with the catheter placed in the carotid artery. After a 7-day recovery period, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded for a 1min period every 60min at an acquisition rate of 1 kHz. The data are presented in two ways. First, to give a view of blood pressure through the 24h cycle, we took an average for each time point for each mouse over 2 consecutive days of recording and used these data to calculate the group mean shown in Figures 1B and 1C, as described (Evans et al., 2016). Second, for each mouse we took a 12h average, making between genotype comparisons for SBP and DBP (Figures 1D and 1E).

Sodium Balance in Conscious Mice: Mice (n=6 per genotype) were housed in metabolic cages with free access to food and water, intake of which was measured daily. Mice were fed 0.25% Na chow for 3 days

03 and then 2.5% Na chow for 6 days. Food intake was used to calculate daily sodium intake. Urine and faeces
04 were collected every 24h and the sodium content of each was combined to provide daily sodium output.
05 Flame photometry (BWB Technologies, UK) was used to measure sodium concentration in urine and the
06 sodium content in dry faeces following extraction in nitric acid (~15 M; Fisher Scientific, UK), as described
07 (Christensen et al., 2015). Cumulative sodium balance (intake-output) was calculated over periods of 3
08 days. In-house, validated enzyme-linked immunosorbent assays were used to measure urinary
09 concentration of aldosterone (Al-Dujaili et al., 2009b), corticosterone and deoxycorticosterone (Al-Dujaili
10 et al., 2009a). For each mouse, measurements were made on urine collected on day 1 and day 3 (baseline)
11 and day 5 and day 6 (high salt). Measurements were averaged used to calculate the group means. 24h
12 urinary steroid excretion was used as an index of global activity of the renin-angiotensin-aldosterone system
13 and hypothalamic-pituitary-adrenal axis.

14 **Renal function:** GR^{βgeo/+} and GR^{+/+} wild-type mice were maintained on either a 0.25% Na or 2.5% Na diet
15 for 7 days and then anaesthetized (Inactin; 120mg/kg; IP) and prepared surgically for renal clearance
16 experiments. Experiments were performed between 9am and 5pm local time. Previous work indicates that
17 under Inactin anaesthesia renal function is similar to that measured in conscious rodents during the active
18 phase (Walter et al., 1989). The carotid artery was cannulated for sampling of arterial blood and the bladder
19 was catheterized for collection of urine. The jugular vein was cannulated for infusion of a solution
20 containing 120mmol/l NaCl, 15mmol/l NaHCO₃ and 5mmol/l KCl. FITC-conjugated inulin (0.25% w:v)
21 and p-aminohippurate (sodium salt, 20mmol/l) were included in the infusate to allow the measurement of
22 glomerular filtration rate (GFR) and renal blood flow (RBF) by clearance methodology, as described
23 (Hunter et al., 2014). After a post-surgery stabilization period, a baseline urine collection was made and
24 then each mouse received an injection of either hydrochlorothiazide (2mg/kg; IV) or benzamil (1mg/kg
25 IV). After 20min, a second urine collection was made. Urine and plasma sodium concentration was
26 measured by ion-selective electrode (9180 Electrolyte Analyser, Roche UK) and used to calculate fractional
27 sodium excretion (FENa), expressed as the percentage of the filtered sodium load excreted in the urine. The
28 net natriuretic response (ΔFENa) to thiazide was used as an index of NCC activity; that of benzamil as an
29 index of ENaC activity, as described (Hunter et al., 2014).

30 **Quantitative PCR.** GR^{+/+} and GR^{βgeo/+} mice were maintained on either a 0.25% or 2.5% sodium diet as
31 above. Following cervical dislocation, the kidneys were snap frozen and mRNA later extracted using Trizol
32 (Invitrogen, UK) and a Purelink RNA mini kit (Qiagen, USA) and quantified using a Nanodrop 1000
33 spectrophotometer (Thermofisher, UK). RNA concentrations were 200-700ng/μl and the A260/280 ratios
34 were >1.7. mRNA abundance was assessed by quantitative PCR. In the first experiment the expression of
35 renal *Nr3c1*, *Nr3c2* and *Hsd11b2* quantified. In the second experiment the expression of *Scnn1a* and
36 *Slc12a3* were measured. Assays were developed using the Universal Probe Library and performed on a
37 LightCycler 480 (both Roche, UK). Each sample was assessed in triplicate and an average value taken. *Tbp*
38 was not different by either diet or genotype and was used here as a control reference gene and all other
39 value were normalized to this expression. qPCR data are expressed relative to GR^{+/+} expression under basal
40 conditions, the group mean of which was set arbitrarily to 100%. The probe number and primer sequences
41 are shown in Table 1.

42 **Statistics:** Data are presented as mean ± standard error or as medians with interquartile range, as
43 appropriate. Statistical comparisons (Graphpad Prism 7 La Jolla, CA) were made by using two-way analysis
44 of variance to assess the main effects of the genotype and diet and the interaction between the main effects.
45 Post-test comparisons were made by using the Holm-Sidak test for multiple comparisons. For sodium and
46 water balance, the non-parametric Kruskal-Wallis test was used, with post-test comparisons being made
47 with Dunn's tests. A p value of <0.05 was considered statistically significant.

48

49 RESULTS

50 ***GR^{βgeo/+} mice have salt-sensitive hypertension***

51 BP was ~10mmHg higher in anesthetized GR^{βgeo/+} mice than in GR^{+/+} mice under normal salt diet of 0.25%
 52 sodium (Figure 1A). This was confirmed in a separate group of conscious, unrestrained mice by
 53 radiotelemetry. SBP (Figure 1B & 1D) was significantly higher (p<0.05) only during the subjective night;
 54 DBP (Figure 1C & 1E) was significantly higher in both day (p<0.05) and night (p<0.01) periods. BP was
 55 next measured in groups of anesthetized mice maintained on a high (2.5% Na) salt diet for 6 days. There
 56 was no effect of salt on BP in GR^{+/+} mice, indicating salt-resistance. In contrast, high salt increased BP by
 57 ~10 mmHg in GR^{βgeo/+} mice (Figure 1A): there was a significant effect of salt (p<0.0001), genotype
 58 (p<0.001) and a significant interaction (p<0.05 interaction).

59 Using quantitative PCR we demonstrated that GR^{βgeo/+} mice had ~50% reduction in renal *Nr3c1* (encoding
 60 GR) mRNA (Figure 2A; Effect of genotype p<0.001). There was no significant effect of 6-days of high salt
 61 diet on renal *Nr3c1* expression. There was also no significant effect of genotype on the renal expression of
 62 *Nr3c2* (encoding MR) but dietary salt increased expression significantly (p<0.01) and to a similar extent in
 63 both groups of mice (Figure 2B). The expression of *Hsd11b2*, which encodes the glucocorticoid
 64 metabolizing enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), was not different between
 65 groups and was not affected by high salt diet (Figure 2C).

66 ***GR^{βgeo/+} mice show transient sodium retention***

67 Sodium balance was measured over periods of 3 days, neutral balance being confirmed in both genotypes
 68 before the high salt challenge. GR^{βgeo/+} mice entered positive sodium balance over the first three days of
 69 2.5% Na diet (Figure 3A), regaining neutral sodium balance during the second 3-day period of high salt.
 70 Wild-type mice remained in neutral sodium balance throughout the 6 days of high salt diet (Figure 3A).
 71 Water balance followed the same profile as that of sodium: GR^{βgeo/+} mice retained more fluid than wild-
 72 types in the first 3 days of the high salt challenge (Figure 3B) and tended to retain more water during days
 73 4-6, but this was not statistically significant.

74

75

76 ***High salt diet increases corticosterone excretion in wild type and GR^{βgeo/+} mice***

77 On 0.25% sodium diet, urinary aldosterone excretion was significantly higher in GR^{βgeo/+} mice than in
 78 controls (Figure 4A; Effect of genotype $p < 0.05$). High salt feeding suppressed aldosterone excretion in both
 79 groups (Effect of salt $p < 0.01$) and excretion was not different between genotypes in this phase of the
 80 experiment (Figure 4A). Basal corticosterone excretion was higher in GR^{βgeo/+} mice (29 ± 2 pmol/day) than
 81 GR^{+/+} (14 ± 1 pmol/day) but this difference was not statistically significant. High dietary salt significantly
 82 increased urinary corticosteroid excretion in both GR^{+/+} and GR^{βgeo/+} mice (Effect of salt $p < 0.001$), with
 83 the effect being more exaggerated in GR^{βgeo/+} mice (Figure 4B; Effect of genotype, $p < 0.05$; Interaction
 84 $p < 0.05$). Excretion of deoxycorticosterone, which is a corticosterone precursor with weak
 85 mineralocorticoid activity, was significantly enhanced by dietary salt in both groups of mice (Figure 4C;
 86 Effect of salt $p < 0.001$), again with a larger effect observed in GR^{βgeo/+} mice (Effect of genotype $p < 0.05$).

87 ***GR^{βgeo/+} mice have impaired renal hemodynamic and tubular adaptation to high salt***

88 Renal clearance experiments were performed on GR^{βgeo/+} and wild-type mice that had been fed either
 89 control or high salt diet for 6 days prior to anesthesia and surgery. Glomerular filtration rate was similar
 90 between genotypes and not affected by dietary salt (Table 2). Under basal conditions renal blood flow
 91 (Figure 5A) and renal vascular resistance (Figure 5B) did not differ between genotypes. High salt diet
 92 doubled renal blood flow in GR^{+/+} mice (Figure 5A; Effect of salt $p < 0.001$); the hyperaemic effect was
 93 attenuated in GR^{βgeo/+} mice and did not achieve statistical significance. Thus, RBF was significantly lower
 94 in high salt-fed GR^{βgeo/+} mice compared to GR^{+/+} controls (Figure 5A; Interaction $p < 0.01$) and renal
 95 vascular resistance, which was reduced by high salt in GR^{+/+} mice, did not change significantly in GR^{βgeo/+}
 96 mice (Figure 5B).

97 FE_{Na} tended to be higher in GR^{βgeo/+} mice than in GR^{+/+} mice on a 0.25% sodium diet, but this was not
 98 significantly different. High salt diet increased FE_{Na} in GR^{+/+} mice (Figure 5C), indicating appropriate
 99 downregulation of tubular sodium reabsorption. In contrast, GR^{βgeo/+} mice, failed to modulate tubular
 00 sodium transport in response to high salt and FE_{Na} was not significantly increased (Figure 5C; Effect of salt
 01 $p < 0.001$; interaction; $p < 0.05$). Impaired renal sodium excretion was not associated with a change in plasma
 02 sodium or potassium (Table 2). Haematocrit was significantly higher in GR^{βgeo/+} mice than controls but

there was no effect of salt diet in either genotype (Table 2). This is difficult to interpret in terms of relative volume status since GR has an important role in erythropoiesis (Bauer et al., 1999)

GR^{βgeo/+} mice do not downregulate NCC or ENaC in response to salt loading

To investigate the molecular mechanisms of altered renal sodium excretion, we focused on the distal nephron, first measuring the mRNA levels of NCC and ENaC, then using the inhibitors thiazide and benzamil to assess protein function *in vivo*.

Slc12a3 mRNA abundance was comparable between genotypes on a standard sodium diet (Figure 6A) and thiazide increased FE_{Na} by 2-3% in both genotypes (Figure 6B), consistent with our previous estimates of *in vivo* NCC transport in C57BL6 mice (Hunter et al., 2014). A 6-day high salt diet did not change *Slc12a3* mRNA expression in GR^{+/+} mice, but thiazide-sensitive sodium transport was significantly reduced, indicating downregulation of functional NCC. In GR^{βgeo/+} mice, high salt feeding significantly increased *Slc12a3* mRNA (Figure 6A): the net-natriuretic response to the thiazide did not increase but was sustained, not downregulated, in high salt-treated GR^{βgeo/+} mice. Overall, the two genotypes had significantly different a natriuretic response to thiazide (Effect of genotype p<0.01).

The expression level of *Scnn1a* mRNA, encoding the alpha subunit of ENaC, did not differ between genotypes (Figure 6C) on 0.25% Na diet. The natriuretic response to benzamil was similar in both groups and indicated that under control salt conditions, ENaC reabsorbed ~0.5% of the filtered sodium load (Figure 6D), consistent with our previous studies for this mouse strain (Bailey et al., 2009). In GR^{+/+} mice, 6 days of high salt diet caused a down-regulation of *Scnn1a* mRNA (Figure 6C) and abolished benzamil-sensitive sodium reabsorption (Figure 6D). This correlation of transcriptional and functional data is consistent with down-regulation of ENaC in response to high salt diet. However, this normal physiological adaptation did not occur in GR^{βgeo/+} mice: Instead, neither *Scnn1a* mRNA abundance (Figure 6C) nor benzamil-sensitive sodium reabsorption significantly reduced with high salt (Figure 6D) and this differential response to dietary salt between genotypes was highly significant (Effect of genotype p<0.001).

27 DISCUSSION

28 We used GR ^{β geo/+} mice to model glucocorticoid resistance, assessing BP and renal sodium handling under
 29 basal and high salt conditions. The modest elevation of basal BP in GR ^{β geo/+} mice was amplified by high
 30 salt intake. Salt-sensitivity was associated with increased levels of deoxycorticosterone and corticosterone,
 31 an abnormal renal haemodynamic response and a failure of the distal nephron sodium to adapt to high salt
 32 intake.

33 *Corticosteroids and salt-sensitive hypertension*

34 Hypertension is a common feature of glucocorticoid excess, often attributed to increased mineralocorticoid
 35 bioactivity, which may occur through three possible routes. First, ACTH induces morphological and
 36 transcriptional changes in the adrenal gland that induce steroidogenesis in the *zona glomerulosa*
 37 (Mazzocchi et al., 1986; Menzies et al., 2017), although the effect on aldosterone production may only be
 38 transient (Dunbar et al., 2010). GR ^{β geo/+} mice have hypertrophied adrenal glands and an expanded *zona*
 39 *glomerulosa* but the elevated circulating aldosterone concentration reflected increased plasma renin
 40 activity, rather than high levels of ACTH (Michailidou et al., 2008). We found enhanced basal urinary
 41 excretion of aldosterone in GR ^{β geo/+} mice but the renin-angiotensin system modulated appropriately with
 42 high salt diet and was not a cause of salt-sensitive BP. Second, transcriptional responses resulting in
 43 corticosterone excess in mice increase production of the biosynthetic intermediate deoxycorticosterone
 44 (Dunbar et al., 2010). In our study, a high salt diet increased deoxycorticosterone excretion, to a greater
 45 extent in GR ^{β geo/+} mice. Deoxycorticosterone is a weak mineralocorticoid but in mice becomes biologically
 46 significant when the HPA axis is chronically activated (Mullins et al., 2009). Indeed, deoxycorticosterone
 47 acetate, in combination with high salt intake, is widely used to induce experimental hypertension in rodents
 48 (Basting and Lazartigues, 2017). Deoxycorticosterone may therefore contribute to salt-sensitive
 49 hypertension in the current study. Third, glucocorticoids can directly activate MR if levels exceed the
 50 inactivating capacity of 11 β HSD2. We found no evidence for altered 11 β HSD2 at the transcript level but
 51 expression of MR was significantly increased by high salt diet to a similar extent in both genotypes. This
 52 was an unexpected finding. One study has also found a positive relationship between dietary salt and MR
 53 expression in rat colon and, to a lesser extent, kidney (Norregaard et al., 2003), but another reports down-

54 regulation of MR by high salt (Lienhard et al., 2012). We do not know why high salt induces MR expression
 55 but importantly in GR^{βgeo/+} mice the increased ratio of MR:11βHSD2 expression was also accompanied by
 56 a 4-fold increase in urinary corticosterone excretion, suggesting that glucocorticoids have the potential to
 57 act as mineralocorticoids in this genetic setting. Indeed, this concept is supported by the analysis of urinary
 58 steroid metabolites in patients with glucocorticoid resistance, which indicates that the 11βHSD2 barrier is
 59 breeched, allowing MR to be activated by cortisol (Bouligand et al., 2010). The expression of serum- and
 60 glucocorticoid-induced kinase-1 (sgk1) has been used to report MR activation in mice (eg (Shibata et al.,
 61 2008)). This was not measured in the current study since sgk1 is also stimulated by GR agonists (Alvarez
 62 de la Rosa et al., 2003) and would not discriminate unequivocally between MR and GR activation in this
 63 mouse model. Indeed, the hypertension of glucocorticoid excess can also involve GR pathways (Nieman et
 64 al., 1985;Bailey et al., 2009).

65 Our study also adds to a growing literature in rodents and humans showing that high salt intake enhances
 66 glucocorticoid bioactivity, either by increasing circulating steroid or by stimulating enzymatic intracellular
 67 amplification (Lewicka et al., 1998;Rakova et al., 2017). We do not know why high salt diet increases
 68 corticosterone. This was not a focus of our study and a systematic analysis of the relationship between
 69 dietary salt and HPA activity is currently lacking. Nevertheless, this positive relationship is exaggerated in
 70 both clinical and experimental models of salt-sensitive hypertension (Liu et al., 2008;Usukura et al.,
 71 2009;Bailey et al., 2011;Craigie et al., 2012;Huesler et al., 2013).

72 ***Vascular dysfunction and salt-sensitive hypertension***

73 Increased salt intake triggers a decrease in systemic vascular resistance in salt-resistant but not salt-sensitive
 74 humans and vascular dysfunction is proposed as a key mechanism of salt-sensitivity (Morris et al., 2016).
 75 Certainly, glucocorticoids influence vascular tone by potentiating the contractile response of resistance
 76 arteries to catecholamines and reducing vasodilatory responses to acetylcholine (Yang and Zhang, 2004).
 77 In our experiment, high-salt feeding to wild-type mice increased renal blood flow and reduced renal
 78 vascular resistance; this response was attenuated in GR^{βgeo/+} mice. A limitation of this study is that our
 79 measurements of renal blood flow were indirect, based on the clearance of p-aminohippurate rather than
 80 direct measurement by Doppler ultrasound. Nevertheless, vascular dysfunction may be an important,

81 causative feature of salt-sensitivity in $GR^{\beta_{geo}/+}$ mice. Certainly other studies suggest this as genetic deletion
 82 of the receptor in the endothelium increases basal blood pressure (Goodwin et al., 2011). Impaired vascular
 83 adaptation to high salt diet may also reflect MR activation, which is detrimental to vascular function in this
 84 setting (Ferrario and Schiffrin, 2015).

85 ***Sodium retention, tubular sodium reabsorption and salt-sensitive hypertension***

86 The switch to high salt diet cause a transient sodium retention in $GR^{\beta_{geo}/+}$ mice yet beyond this point, when
 87 neutral sodium balance was regained, we found a persistent enhancement of tubular sodium reabsorption.
 88 This may seem incompatible but restoration of sodium balance is of major homeostatic importance and will
 89 occur despite underlying tubular abnormalities that impair the acute pressure natriuresis response (Hall,
 90 2016, Ivy & Bailey, 2014). Thus, sodium excretion can match intake over period of 24h even if there is a
 91 reduced ability to excrete sodium rapidly. Such delayed attainment of balance impacts BP, disturbing
 92 normal circadian rhythm (Bankir et al., 2008) and causing salt-sensitivity (Staessen et al., 1993), as seen in
 93 $GR^{\beta_{geo}/+}$ mice. To identify the molecular mechanism of altered tubular sodium transport, we focused on
 94 the major apical sodium transporters in the aldosterone-sensitive distal nephron, NCC and ENaC (Bailey,
 95 2016), comparing measurements of mRNA with an *in vivo* assessment of transporter function. We did not
 96 measure protein abundance or distribution, a limitation to our study, but for ENaC, the mRNA and
 97 pharmacological data gave a consistent message: ENaC, which normally downregulates at both mRNA and
 98 protein levels with high salt intake (Udwan et al., 2017), did not downregulate in $GR^{\beta_{geo}/+}$ mice despite
 99 reduced aldosterone. Sustained ENaC-mediated sodium reabsorption could be attributed to a salt-induced
 00 increase in deoxycorticosterone and/or corticosterone. Glucocorticoids can activate ENaC via GR (Schulz-
 01 Baldes et al., 2001; Bailey et al., 2009; Nguyen Dinh Cat et al., 2009; Craigie et al., 2012), but here it is more
 02 likely that the 11β HSD2 barrier is exceeded (Bouligand et al., 2010) and ENaC is activated via MR, as
 03 discussed above.

04 For NCC, mRNA and thiazide-sensitivity did not directly correlate but were directionally coherent. Thus,
 05 in $GR^{\beta_{geo}/+}$ mice fed a high-salt diet, mRNA and thiazide-sensitive sodium transport were both higher than
 06 in $GR^{+/+}$ mice. Why this occurs is not certain. High salt diet normally reduces total NCC protein and
 07 phosphorylation at site important for trafficking to the apical membrane of the distal convoluted tubule cell

(Udwan et al., 2017). In GR^{βgeo/+} mice, thiazide-sensitive sodium reabsorption was not reduced by high salt diet but we do not know if this reflects an increase in the total NCC protein pool, as suggested by mRNA, and/or a change in the proportion of NCC that is phosphorylated. The distal convoluted tubule expresses MR and GR (Ackermann et al., 2010) but there is no expression of 11βHSD2 in the early part and only low levels towards the end of this segment (Hunter et al., 2015; Ivy et al., 2016). Chronic aldosterone excess can increase NCC activity (Ashek et al., 2012; Poulsen and Christensen, 2017) but this is secondary to mineralocorticoid-induced hypokalemia (Czogalla et al., 2016) and high-salt diet did not induce hypokalaemia in GR^{βgeo/+} mice. A GR-mediated response is also possible since dexamethasone increases expression (Chen et al., 1994) and *in vivo* activity of NCC (Velazquez et al., 1996). However, given the reduced receptor expression in GR^{βgeo/+} mice, the importance of this pathway is uncertain.

In summary, this study shows that a global reduction in GR expression induces a pro-hypertensive relationship between blood pressure and dietary salt intake in mice, mostly likely reflecting MR activation by glucocorticoids. This provides mechanistic insight into the importance of genetic and environmental interactions in determining cardiovascular risk.

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84

Figure Legends

Figure 1: Mean Arterial Pressure (A) was measured acutely in $GR^{+/+}$ (open squares) or $GR^{\beta_{geo}/+}$ (red squares) littermates. Mice were fed either 0.25% sodium (n=19/15) or 2.5% sodium diet (n=15/17). Data are provided for individual mice, along with mean \pm sem for each group, and comparisons were made by two-way ANOVA with post hoc Holm-Sidak tests for defined comparisons. (B-C) In a second group of conscious, unrestrained mice, blood pressure was measured by radiotelemetry. $GR^{+/+}$ (black line; n=6) and $GR^{\beta_{geo}/+}$ (red line; n=4), mice. Data are group mean generated by averaging each mouse over 2 consecutive days of recording, dotted lines indicate SEM. ZT = 0 was 0700h local time. Lights were on from ZT 0-12 and mice were asleep during this subjective day. The shaded area represents the period of subjective night, when mice were active.

Figure 2: mRNA expression of (A) *Nr3c1*, encoding GR; (B) *Nr3c2*, encoding MR; and (C) *Hsd11b2*, encoding 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2) in $GR^{+/+}$ (open squares; n=7/7) and $GR^{\beta_{geo}/+}$ mice (red squares; n=6/6) on 0.25% sodium or a 2.5% sodium diet. Data from individual mice are presented, along with the group mean \pm SEM. Data were analysed by two-way ANOVA with post hoc Holm-Sidak tests. Post-test comparisons of the effect of a high salt within genotype are shown *p<0.05. Between-genotype post-test comparisons are as indicated.

Figure 3: Sodium (A) and water balance (B) in $GR^{+/+}$ (n=5; open boxes) and $GR^{\beta_{geo}/+}$ (n=6; red boxes) mice on a 0.25% sodium diet and then a 2.5% sodium diet. Cumulative sodium and water balance is displayed in 3-day bins at baseline (first pair of bars) and during adaptation to a high sodium diet (the second pair of bars represent Na balance over days 1-3; the third set days 4–6 of high sodium feeding). Data are median and range and were analysed by non-parametric Kruskal-Wallis tests, with post hoc Dunn's tests.

11 **Figure 4:** 24-h urinary excretion of (A) aldosterone, (B), corticosterone and (C) deoxycorticosterone in
 12 GR^{+/+} (n=5; open squares) and GR ^{β geo/+} (n=6; red squares) mice on 0.25% sodium and then 2.5% Na diet.
 13 The 3-day average for each mouse is shown along with the group mean \pm SEM. Data were analysed by two-
 14 way ANOVA with post hoc Holm-Sidak tests. Post-test comparisons of the effect of a high salt within
 15 genotype are shown as *p<0.05, **p<0.01, ***p<0.001. Between-genotype post-test comparisons are as
 16 indicated.

17
 18 **Figure 5:** A) Renal blood flow, B) renal vascular resistance and (C) fractional sodium excretion in GR^{+/+}
 19 (open squares) and GR ^{β geo/+} (red squares) mice maintained on either a 0.25% sodium or 2.5% Na diet. Data
 20 from individual mice are presented as are the group mean \pm SEM. Data were analysed by two-way ANOVA
 21 with post hoc Holm-Sidak tests. Post hoc comparisons of the effect of a high salt within genotype are shown
 22 ***p<0.001. Between-genotype post hoc comparisons are as indicated.

23
 24 **Figure 6:** Sodium transporter mRNA abundance and *in vivo* activity in GR^{+/+} (open squares) and GR ^{β geo/+}
 25 mice (red squares) on 0.25% sodium or a 2.5% sodium diet. A) mRNA expression of *slc12a3*, encoding
 26 NCC; B) the net natriuretic response to thiazide, taken to reflect NCC-mediated sodium reabsorption; C)
 27 mRNA expression of *scnn1a*, encoding α subunit of ENaC; D) the net natriuretic response to benzamil,
 28 taken to reflect ENaC-mediated sodium reabsorption. AU = arbitrary units. Data from individual mice are
 29 presented, along with the group mean \pm SEM. Data were analysed by two-way ANOVA with post hoc Holm-
 30 Sidak tests. Post-test comparisons of the effect of a high salt within genotype are shown *p<0.05. Between-
 31 genotype post-test comparisons are as indicated.

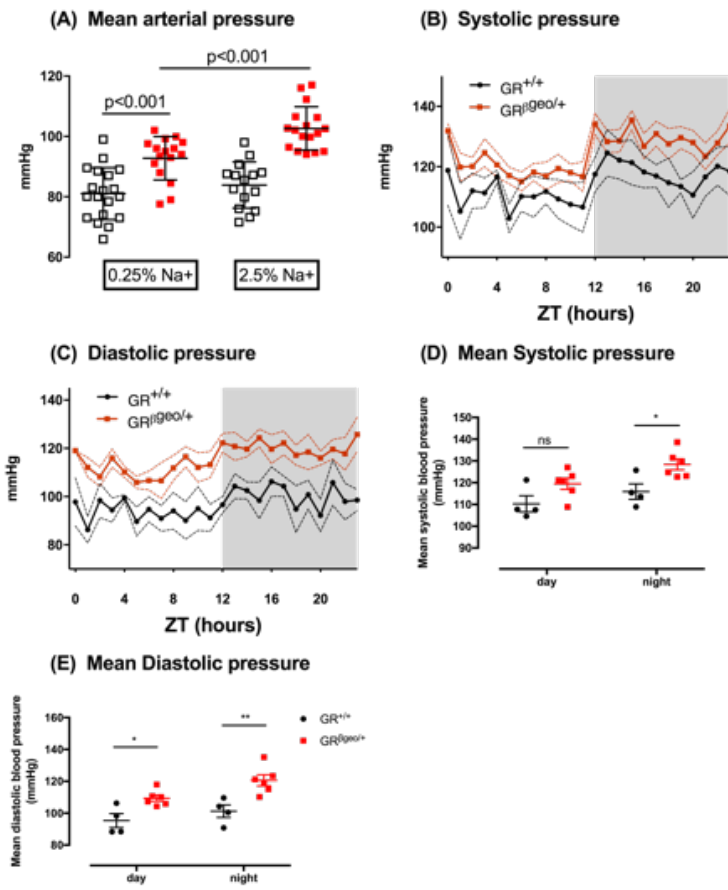
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Table 1: Primers for qPCR. The genes encode the following proteins: TATA-binding protein, glucocorticoid receptor, mineralocorticoid receptor, 11 β -hydroxysteroid dehydrogenase type 2, the thiazide-sensitive co-transporter and the alpha subunit of the Epithelial Sodium Channel.

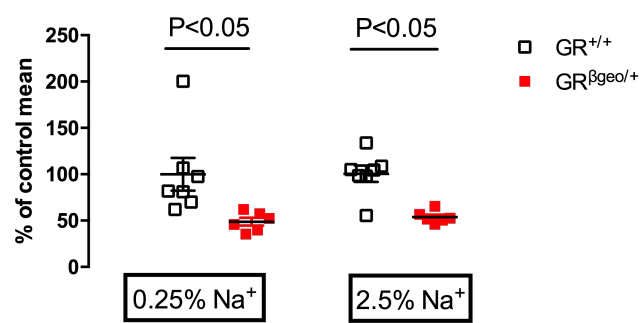
Gene	Forward Sequence	Reverse Sequence	Probe Number
<i>Tbp</i>	gggagaatcatggaccagaa	gatgggaattccaggagtca	97
<i>Nr3c1</i>	caaagattgcaggtatcctatgaa	cttggtcttcagaccttc	91
<i>Nr3c2</i>	caaaagagccgtggaagg	tttctccgaatcttatcaataatgc	11
<i>hsd11b2</i>	cactcgaggggacgtattgt	gcaggggtatggcatgtct	26
<i>slc12a3</i>	cctccatcaccaactcacct	ccgcccacttgctgtagta	12
<i>scnn1a</i>	ccaagggtgtagagtctgtga	agaaggcagcctgcagtta	45

Table 2: Glomerular Filtration Rate (GFR), plasma sodium and potassium concentration and haematocrit in GR^{+/+} and GR ^{β geo/+} mice maintained on either 0.25% (n=15/16) or 2.5% sodium diet (n=17/19) for 7 days. Data are mean \pm SEM and were analysed by two-way ANOVA with post hoc Holm-Sidak tests, ⁺p<0.05, +++p<0.001 significant effect of high salt diet within a given genotype, ***p<0.001 **p<0.01 significant effect of genotype within a given diet.

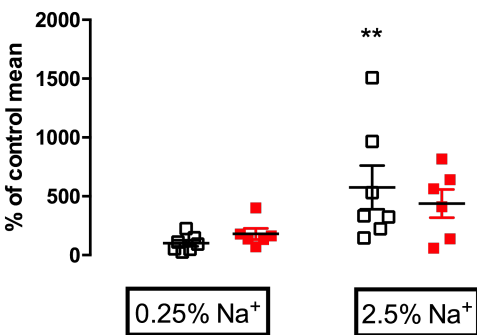
	0.25 % Na ⁺		2.5% Na ⁺	
	GR ^{+/+}	GR ^{βgeo/+}	GR ^{+/+}	GR ^{βgeo/+}
GFR (ml/min)	0.20 \pm 0.02	0.20 \pm 0.02	0.25 \pm 0.02	0.22 \pm 0.02
Plasma Na (mmol/l)	145.7 \pm 1.0	144.2 \pm 0.9	145.3 \pm 0.9	144.6 \pm 1.1
Plasma K (mmol/l)	4.4 \pm 0.2	4.2 \pm 0.1	4.5 \pm 0.2	4.5 \pm 0.2
Haematocrit (%)	42.7 \pm 1.0	<u>45.0 \pm 0.9***</u>	44.0 \pm 0.3	<u>46.8 \pm 0.7***</u>



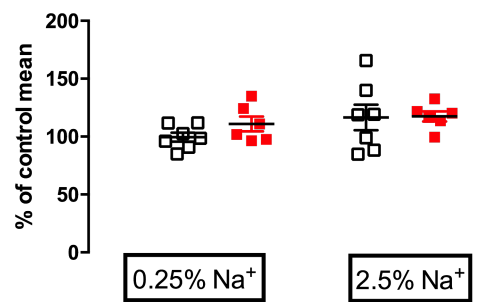
A) GR expression

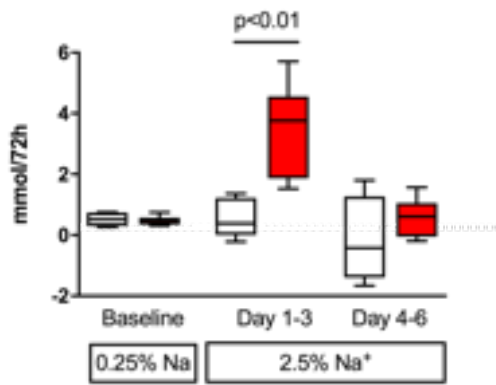
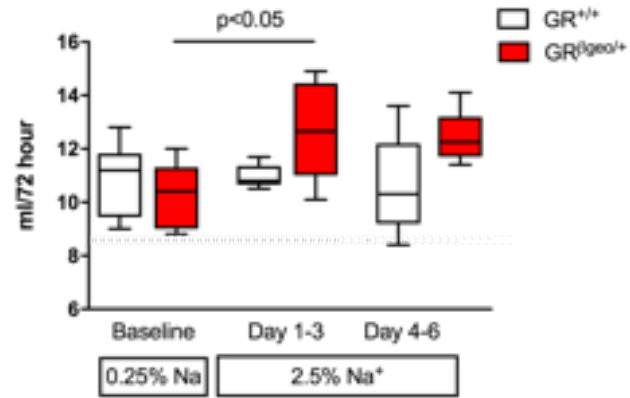


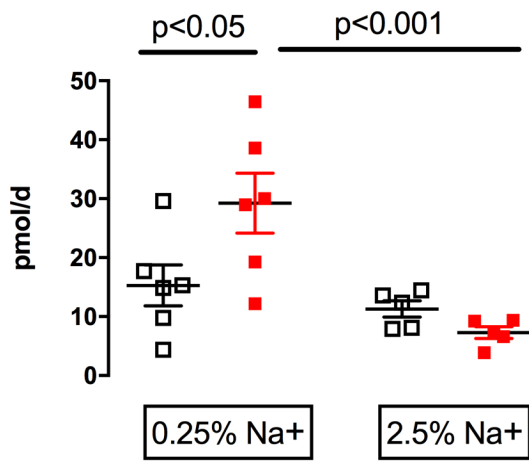
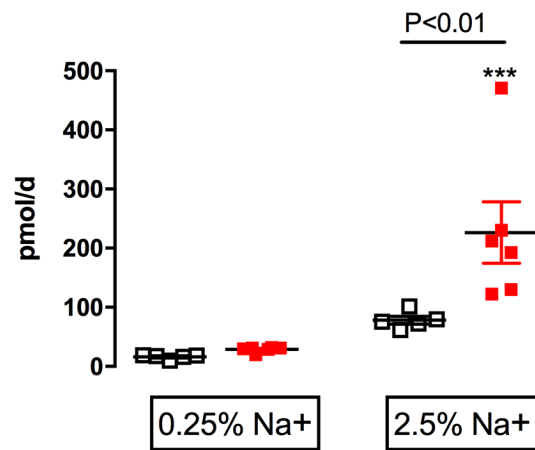
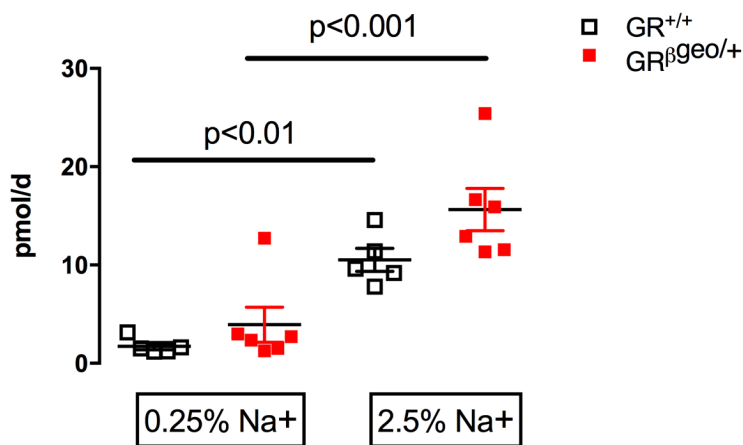
B) MR expression



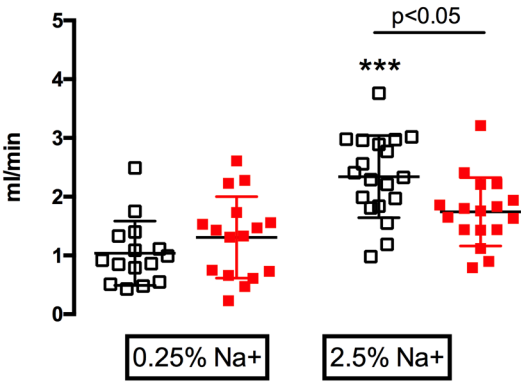
C) 11βHSD2 expression



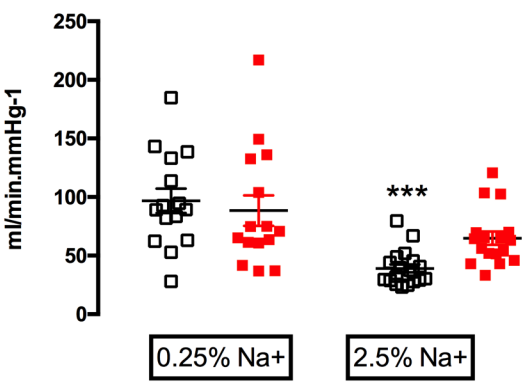
(A) Sodium balance**(B) Water balance**

(A) Aldosterone excretion**(B) Corticosterone excretion****(C) Deoxycorticosterone excretion**

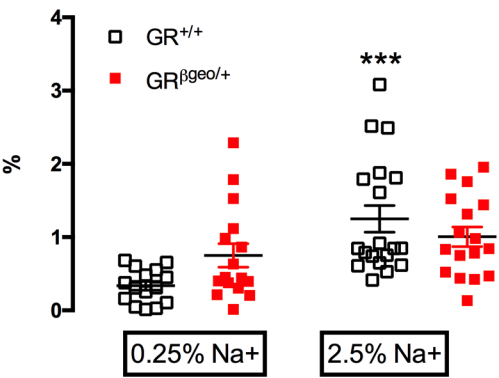
(A) Renal blood flow



(B) Renal vascular resistance

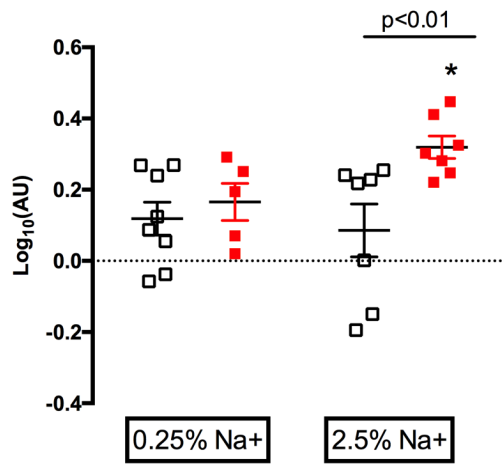
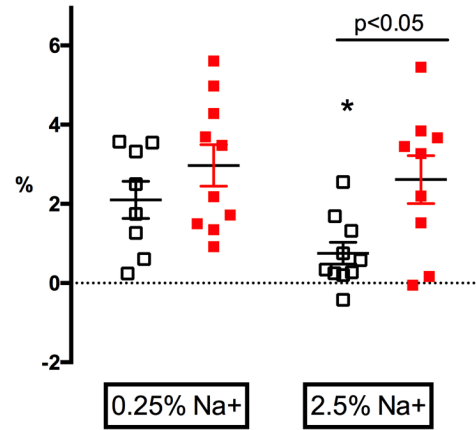
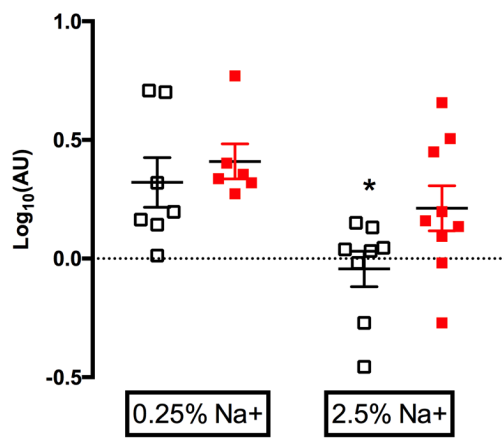
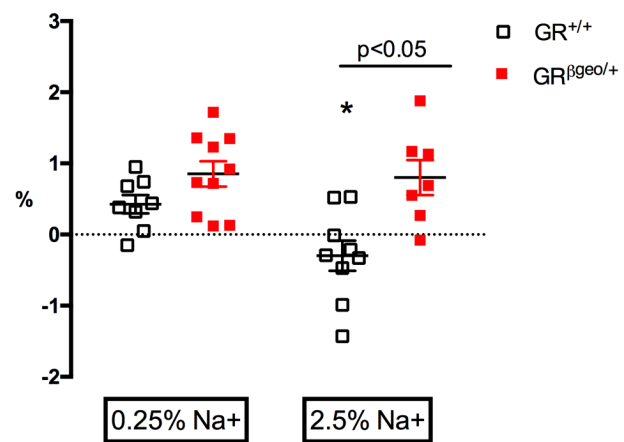


(C) Fractional sodium excretion



51

52

(A) *Slc12a3* mRNA**(B) NCC-mediated FE_{Na}****(C) *Scnn1a* mRNA****(D) ENaC-mediated FE_{Na}**

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54